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Short communication

# A combination of two lactic acid bacteria improves the hydrolysis of gliadin during wheat dough fermentation

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ABSTRACT

The evaluation of gliadin hydrolysis during dough fermentation by using two lactic acid bacteria, *Lactobacillus plantarum* CRL 775 and *Pediococcus pentosaceus* CRL 792, as pooled cell suspension (LAB) or cell free extract (CFE) was undertaken. The CFE pool produced a greater (121%) increase in amino acid concentration than the LAB pool (70–80%). These results were correlated with the decrease (76,100 and 64,300 ppm) in the gliadin concentration of doughs supplemented with CFE and LAB, respectively, compared to control doughs. The use of LAB peptidases seemed to be a viable technologic alternative to reduce the gliadin concentration in wheat dough without using living bacteria as starter.



## 1. Introduction

Wheat gluten proteins are among the most studied of food proteins. They are divided according to their solubility properties into two major groups: gliadins and glutenins, the first one being involved in the celiac disease (Sollid and Khosla, 2005). Several residues of the N-terminus of α-gliadin are immunologically active, e.g., amino acids at position 31–43, 62–75 and 57–89 in the protein (Gerez et al., 2008) causing an inflammatory response of the small intestinal mucosa (Sturgess et al., 1994; Arentz-Hansen et al., 2000; Shan et al., 2002). The large proportion and location of proline (P) residues in these toxic peptides make them extremely resistant to proteolysis (Stepniak et al., 2006), thus specific peptidases are necessary to hydrolyze the cyclic structure of P (Hausch et al., 2002).

The proteolytic system of lactic acid bacteria (LAB) of sourdough origin has been subjected to several studies from a nutritional and technological standpoint, where the ability of LAB to hydrolyze α-gliadin-fragments is in a relevant place (De Angelis et al., 2005; Sollid and Khosla, 2005; Rollán et al., 2005; Gerez et al., 2008). In a previous study, several lactobacilli and pediococci were tested for the ability to hydrolyze the 31–43, 62–75, and 57–89 (33 mer) α-gliadin fragments; the latter one was only degraded (56.6%) by the pool *Lactobacillus plantarum* CRL 775/*Pediococcus pentosaceus* CRL 792 (Gerez et al., 2008). These results are relevant considering that the 33 mer fragment is regarded as one of the main peptides involved in the inflammatory response to gluten (Shan et al., 2002).

The aim of this study was to evaluate the gliadin hydrolysis during wheat dough fermentation by the pool *L. plantarum* CRL 775/*P. pentosaceus* CRL 792.

## 2. Materials and methods

### 2.1. Microorganisms and growth conditions

*Lactobacillus* (*L.*) *plantarum* CRL 775 and *Pediococcus* (*P.*) *pentosaceus* CRL 792 was isolated from homemade wheat sourdough and belong to the culture collection of Centro de Referencia para Lactobacilos (CERELA-CONICET, Tucuman, Argentina). The LAB strains were grown in MRS broth (Oxoid) at 37 °C for 16 h; cells were harvested by centrifugation (7000 g, 15 min, 4 °C), washed twice with 10 mmol/l potassium phosphate buffer (pH 7.0), suspended (20% w/v) in the same buffer and disrupted with a French press (French Pressure Cells Thermo Spectronic, USA). The cell-free extracts (CFE) obtained by centrifugation (9000 g, 15 min, 4 °C) were mixed (1:1) and used for enzymatic assays. The concentration of protein in the CFE was determined (Bradford, 1976) using bovine serum albumin as standard and standarized to 5 mg/ml protein.

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### 2.2. Peptidase activities of L. plantarum CRL 775 and P. pentosaceus CRL 792

Prolyl iminopeptidase (PepI), gluteryl aminopeptidase (PepA), X-prolyl-dipeptidyl aminopeptidase (PepX), prolidase (PepQ) and endopeptidase activities were determined (Rollán et al., 2005). One unit of enzyme was defined as the amount of enzyme required to release 1 μmol of p-nitroaniline per minute under the assay conditions.

### 2.3. Dough fermentation

One hundred grams of commercial wheat flour 000 type (71.1% carbohydrates, 2.8% fiber, 10% protein), 45 ml tap water and commercial baker's yeast (*Saccharomyces cereviciae*) as leaven agent were used for preparing the doughs. The doughs supplemented with the pool CRL 775/CRL 792 (*L. plantarum* CRL 775/*P. pentosaceus* CRL 792) (ratio 1:1; 15 ml, v/v) as either cell suspension (LAB) (final concentration = $10^9$ cfu/g) or CFE (5 mg/ml protein) were named as $D_{LAB}$ and $D_{CFE}$, respectively. Doughs (without LAB or CFE) acidified ($D_a$) to pH 4.0 with lactic acid and acetic acid (ratio 4:1) or not acidified ($D_c$) were used as control. The dough fermentation was carried out in flasks at 30 °C, and samples (10 g) were drawn aseptically at 0, 4, 8 h and at the end of the incubation time (24 h). Fermentation and dough analysis were carried out as triplicate assays. The dough pH was measured with a pH meter (Altronix-TPX1 pH/mV-Meter, Argentina).

Samples of fermented doughs (10 g) were homogenized with distilled water (90 ml) in a homogenizer (The Virtis Company, Gardiner, NY) and centrifuged at 8000 g for 10 min. Cell viability, pH, and amino acid concentration were determined in the dough supernatants. The viability of LAB and yeast was determined by the plate dilution method using MRS agar (Oxoid) with cycloheximide (200 g/ml) for the LAB strains, and yeast peptone dextrose agar medium (YPD) with chloramphenicol (500 g/ml) for the Baker yeast. The plates were incubated for 48 h at 37 °C under microaerophilia conditions, and at 30 °C, respectively. Results were expressed as log cfu/g. The total amino nitrogen was determined by Cd-ninhydrin method (Doi et al., 1981). Glycine solution (1 mM) was used as a standard and results were expressed as mmol of glycine per kilogram of dough.

### 2.4. Determination of gliadin in the doughs

Gliadin content of the doughs was determined by Elisa Test [R5-sandwich enzyme-linked immunoabsorbent assay (Ridascreen Gliadin kit, R-Biopharm AG, Germany)] (Collin et al., 2004). The test uses the R5 antibody which is specific for amino acid sequences such as such as QQPFP, LQLQPFP, QLPYP, PQPF and PQPFP (Osman et al., 2001; Kahlenberg et al., 2003) that are present in gliadins. The dough samples (1 g) were placed in 10 ml ethanol 60% (v/v) and incubated at room temperature for 30 min in a rotary shaker (45 rpm). After centrifugation (2500 g, 10 min) the supernatants were immediately analyzed according to manufacturer's instructions.

### 2.5. Statistical analysis

Results presented are the mean values ± standard deviation (SD) of three independent assays. Data were compared by ANOVA and Dunnett *t*-test. The statistical significance ($P < 0.05$) was determined by using Minitab-12 software.

## 3. Results and discussion

The combination of *L. plantarum* CRL 775 and *P. pentosaceus* CRL 792, strains selected by their ability to degrade the 33 mer fragments *in vitro* (Gerez et al., 2008), was used in this study. Gliadins are characterized by a large proportion of proline residues; the proline-specific peptidase and endopeptidase activities of the cell-free extracts pool (CFE 775/792) were characterized by using proline specific synthetic substrates. Results are shown in Table 1. The CFE 775/792 displayed different proline-specific enzymes such as prolyl iminopeptidase (PepI), X-prolyl-dipeptidyl aminopeptidase (PepX) and prolidase (PepQ) as well as gluteryl aminopeptidase (PepA) and endopeptidases (EPa and EPb) activities. The PepI, PepX and PepQ activities, considered key enzymes for degradation of P-rich gliadin polypeptides (Gallo et al., 2005; De Angelis et al., 2010) were two times higher than those reported for combinations of the CFE of four lactobacilli (*Lactobacillus alimentarius* 15M, *Lactobacillus brevis* 14G, *Lactobacillus sanfranciscenis* 7A, y *Lactobacillus hilgardii* 51B) (Di Cagno et al., 2002).

The pool *L. plantarum* CRL 775 and *P. pentosaceus* CRL 792 was evaluated in dough assays as cell suspension ($D_{LAB}$) or as CFE ($D_{CFE}$). Wheat endogenous enzymes such as proteinase and carboxipeptidase (Bleukx and Delcour, 2000; Capocchi et al., 2000) are also able to degrade proline-rich peptide under acid conditions (pH 3.0–4.5). To confirm this hypothesis, acidified control doughs ($D_a$) were included in the assay. All doughs were incubated during 24 h at 30 °C. Results are shown in Table 2.

The LAB counts increased in $D_{LAB}$ by 1.43 log units producing a decrease in pH from 5.5 to 4.3 and to 3.9 after 4 h and 8 h fermentation, respectively, which was not observed for the other doughs. On the whole, the yeast population remained almost unchanged up to 8 h fermentation except for $D_a$ doughs where a lost in cell viability (1.13 log units) was observed. In contrast, the yeast growth was not affected in $D_{LAB}$ doughs despite the low pH reached at the end of fermentation. A possible explanation for this different behavior is the lower acetic acid concentration determined in $D_{LAB}$ (5 mmol/kg) respect to $D_a$ (21 mmol/kg). The acetic acid is more lethal to yeast cells than lactic acid (Garay-Arroyo et al., 2004; Graves et al., 2007) and this toxicity is maximal at low pH (Gerez et al., 2009). On the other hand, the slow development of acidity during LAB growth in $D_{LAB}$ doughs, which would enable the yeast to adapt to the new changing environment compared to the low pH that the cells encountered in $D_a$ doughs from the beginning of fermentation.

The proteolysis in doughs is shown in Fig. 1. The concentration of amino nitrogen increased in all doughs within 24 h fermentation. At this time, the amino acids concentration in $D_c$, $D_a$ and $D_{LAB}$ 775/792 was about 7.2 mmol glycine/Kg dough ($P > 0.05$) while in $D_{CFE}$ 775/792 doughs increased to 9.8 mmol glycine/Kg dough. These results evinced that the CFE pool was an effective source of

**Table 1**
Peptidase activities of the pool *L. plantarum* CRL 775/*P. pentosaceus* CRL 792 on chromogenic substrates.

| Peptidase enzymes | Enzymatic activities[a] |
|---|---|
| PepI | 2.0 ± 0.7 |
| PepX | 36.1 ± 6.4 |
| PepA | 2.7 ± 0.9 |
| PepQ | 2.7 ± 0.9 |
| EPa | 1.2 ± 0.3 |
| EPb | 2.3 ± 0.2 |

PepI: prolyl iminopeptidase, PepX: X-prolyl-dipeptidyl aminopeptidase, PepA: gluteryl aminopeptidase, PepQ: prolidase, EPa: endopeptidase on N-succinyl-L-phenyl-alanine-p-NA and EPb: endopeptidase on N-glutaryl-L-phenyl-alanine-p-NA.
[a] μmol/min.mg prot.

**Table 2**
Evolution of pH, lactic acid bacteria and yeast during dough fermentation.

| Doughs | Time (h) | LAB[a] | Yeast[a] | pH |
|---|---|---|---|---|
| $D_c$[b] | 0 | 4.23 ± 0.13 | 7.06 ± 0.03 | 5.7 ± 0.03 |
| | 4 | 4.45 ± 0.26 | 7.45 ± 0.04 | 5.4 ± 0.03 |
| | 8 | 4.53 ± 0.06 | 7.60 ± 0.43 | 5.3 ± 0.03 |
| | 24 | 4.56 ± 0.05 | 6.28 ± 0.46 | 5.1 ± 0.03 |
| $D_a$[c] | 0 | 4.52 ± 0.09 | 7.13 ± 0.01 | 4.0 ± 0.03 |
| | 4 | 4.63 ± 0.30 | 6.51 ± 0.11 | 4.0 ± 0.03 |
| | 8 | 4.53 ± 0.53 | 6.00 ± 0.18 | 4.0 ± 0.03 |
| | 24 | 4.23 ± 0.28 | 5.92 ± 0.15 | 4.0 ± 0.05 |
| $D_{LAB}$ | 0 | 7.17 ± 0.04 | 7.09 ± 0.03 | 5.5 ± 0.03 |
| | 4 | 8.60 ± 0.07 | 7.35 ± 0.17 | 4.3 ± 0.03 |
| | 8 | 8.62 ± 0.10 | 7.57 ± 0. 09 | 3.9 ± 0.03 |
| | 24 | 8.68 ± 0.11 | 6.91 ± 0.14 | 3.8 ± 0.03 |
| $D_{CFE}$ | 0 | 4.23 ± 0.28 | 7.02 ± 0.01 | 5.7 ± 0.03 |
| | 4 | 4.34 ± 0.41 | 7.47 ± 0.23 | 5.4 ± 0.03 |
| | 8 | 4.62 ± 0.53 | 7.62 ± 0.11 | 5.3 ± 0.03 |
| | 24 | 4.68 ± 0.25 | 7.11 ± 0.12 | 5.0 ± 0.03 |

[a] Colony counts (LAB and yeast) expressed as log cfu/g dough.
[b] Dc: Control dough prepared without the pool CRL 775/792. 775/792: Mixture of *L. plantarum* CRL 775/*P. pentosaceus* CRL 792 (ratio 1:1) as cell suspension ($D_{LAB}$) or cell free extract ($D_{CFE}$).
[c] Da: chemically acidified control dough.

peptidases enzymes which hydrolyzed the wheat flour peptides and enriched the dough with amino acids, thus increasing the nutritional value or improving the texture and aroma of bread (Zotta et al., 2006).

The effect of the pool CRL 775/792 on the α-gliadin content was determined and compared with control doughs ($D_c$ and $D_a$). The pool CRL 775/CRL 792 was used in two modalities: as cell biomass ($D_{LAB}$) and as cell-free extract ($D_{CFE}$) containing proteolytic enzymes. The gliadins concentration showed a steady decrease in doughs $D_{LAB}$ and $D_{CFE}$ during fermentation. After 24 h, the gliadin hydrolysis was 70.3 and 82.7%, respectively, while it was limited (*ca.*14%) in $D_c$ for the same period (Fig. 2). Comparing both modalities, the degradation of gliadins was lower in $D_{LAB}$ with respect to those supplemented with the enzyme extracts ($D_{CFE}$). The lower gliadin degradation obtained with the LAB pool ($D_{LAB}$) compared to CFE may be explained by the fact that the peptides have to be transported across the cell membrane into the cytoplasm and then degraded by specific intracellular peptidases. In contrast, the gliadin hydrolysis in $D_{CFE}$ dough seems to occur immediately after the free enzymes cocktail encountered the gliadin.

In the $D_c$, the concentration of gliadins remained unchanged (*ca.* 79,100 ppm) up to 8 h incubation compared to $D_a$ doughs where the gliadins content decreased by 45% (about 42,900 ppm) within the first 4 h incubation. This fact may be ascribed to wheat endogenous

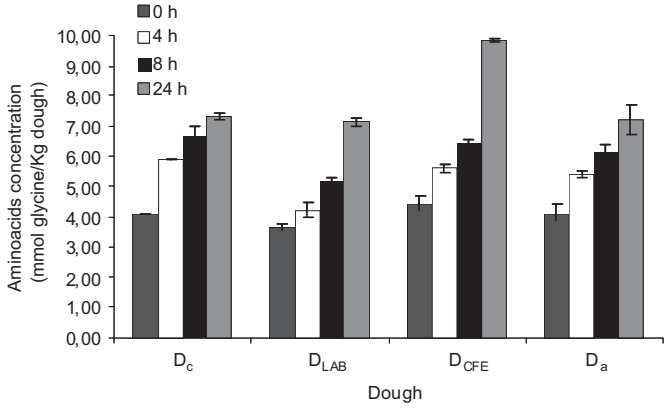


**Fig. 1.** Amino acids concentration of doughs. Control dough ($D_c$); chemically acidified dough ($D_a$); doughs supplemented with pooled *L. plantarum* CRL 775/*P. pentosaceus* CRL 792 as cell suspension ($D_{LAB}$) and cell free extract ($D_{CFE}$).

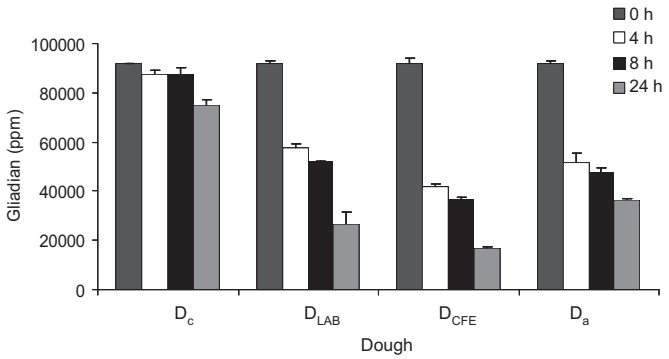


**Fig. 2.** Gliadins concentration in doughs. Control dough ($D_c$); chemically acidified dough ($D_a$); doughs supplemented with pooled *L. plantarum* CRL 775/*P. pentosaceus* CRL 792 as cell suspension ($D_{LAB}$) and cell free extract ($D_{CFE}$).

proteinase enzymes that are activated at low pH (Bleukx and Delcour, 2000). After 8 h fermentation, doughs $D_{LAB}$ presented similar acid conditions (pH 3.9) for activation of endogenous peptidades, which would participate together with the LAB pool in the gliadin hydrolysis. In contrast, the flour endogenous enzymes were inactive in doughs $D_{CFE}$ where the pH remained above 5.0 throughout the incubation period (Capocchi et al., 2000). In this case, the gliadin degradation (about 82.7%) was only due to the activity of the cytoplasmic enzymes pool (CFE 775/792).

In terms of bread elaboration, the chemical acidification of doughs as a tool for reducing the concentration of gliadins by means of wheat flour proteolytic enzymes activation (optimum pH 3.0–4.5) is not feasible from a technological standpoint. The activity of these enzymes depends on many different variables such as the type of flour, the wheat species and the climate conditions of crops, among others (Loponen et al., 2006). Besides, the dough obtained by this way had a strong acid taste and a low yeast count that affects the dough leavening (Capocchi et al., 2000).

The gliadins degradation obtained with the pool of either LAB strains ($D_{LAB}$) or cytoplasmic content ($D_{CFE}$) was higher (1.5 time) than values reported for proteolytic lactobacilli. Rizzello et al. (2007) obtained a reduction in gliadin of 27.000 ppm by using combinations of six *Lactobacillus sanfranciscensis* strains (LS3, LS10, LS19, LS23, LS38 and LS47) and a decrease of 31,000 ppm by using a combination of four strains of different species (*L. alimentarius* 15M, *L. brevis* 14G, *L. sanfranciscenis* 7A, and *L. hilgardii* 51B) in wheat sourdough fermentation at 48 h. Other authors (Loponen et al., 2007; M'hir et al., 2008) reported even lower (14,700–25,000 ppm) gliadin hydrolysis using strains of *L. brevis* and *Enterococcus faecalis*.

Within gliadin peptides, the 33 mer fragment is one of the most resistant to degradation by proteolytic enzymes. Lactobacilli have a very complex peptidase system (Savijoki et al., 2006) but a single strain may not have the entire peptidases profile that are needed for hydrolyzing all potential gluten polypeptides (Gobbetti et al., 2007). In agreement, no single LAB strain was active against 33 mer *in vitro* assays but a pool of *L. plantarum* CRL 775 and *P. pentosaceus* CRL 792 which hydrolyzed it (60%) after 24 h incubation (Gerez et al., 2008). The activities PepI, PepX and PepQ peptidases present in the pool are considered key enzymes for degradation of P-rich gliadin polypeptides (Gallo et al., 2005; De Angelis et al., 2010).

The concept of complete elimination of gluten is controversial. Gluten is considered essential for wheat baking and the complete elimination of gluten from wheat, albeit possible, is technically challenging in industrial baking operations. The controlled proteolysis in wheat doughs was suggested to reduce gliadin levels to an extent that these products are tolerated by celiac patients (Rizzello

et al., 2007; De Angelis et al., 2010). However, the reduced-gluten doughs with extended fermentation time are not suitable for bread production. Hence, the reduced-gluten doughs only can be incorporated as baking improvers into gluten-free bread with quality inferior when compared to conventional (wheat) products (Arendt-Hansen et al., 2005; Rizzello et al., 2007) or used in the manufacture of certain products, like cookies, cakes and pastries. With respect to this last product, the formulation of an Italian pasta made with allergenic (wheat or rye) combined with non-allergenic flours (millet flour and oat species genetically developed partly free and wheat gluten) and fermented by a combination of LAB (*L. alimentarius* 15M, *L. brevis* 14G, *L. sanfranciscenis* 7A, and *L. hilgardii* 51B) has been reported (Di Cagno et al., 2004, 2005). However, the product obtained at the end of fermentation (24 h) has a concentration of 500 ppm gliadin, which is higher than permitted in foods for celiacs. In this work, the use of LAB peptidases (e.g., the CFE pool of *L. plantarum* CRL 775 and *P. pentosaceus* CRL 792) seemed to be a viable technologic alternative to reduce the gliadin concentration in wheat dough without using living bacteria as starter. However, the concentration (~14,800) of gliadins remaining in the dough added with the pool CFE 775/792 is still high according to values permitted in foods for celiac patients (Gallagher et al., 2004). Another possibility to achieve food products for celiac patients would be to combine different strategies, e.g., mixtures of wheat and non-allergenic flours (rice, soybeans or corn) (Arendt-Hansen et al., 2005; Di Cagno et al., 2008) together with the selected *L. plantarum* CRL 775/*P. pentosaceus* CRL 792 pooled (CFE) and fungal proteases (Rizzello et al., 2007).

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